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Antibacterial Activity of Fermentation Broth of Various Actinomycetes Strains and Enhancement of Broth Activity through UV Induced Mutagenesis

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ABSTRACT

Strain improvement programs involving random mutation and subsequent screening for higher-producing progeny is important in applied microbiological research, especially in the production of clinically important antibiotics to enhance the productivity. The present research work dealt with some selected strains of actinomycetes which were subjected to antibiotics production via shake-flask fermentation after being exposed to UV radiation at various time intervals. Some of the mutated strains demonstrated lesser turbidity then that of wild strain and other mutated strains which may be attributed to the harmful effects of UV light. These samples further didn't show any activity against test microorganisms when evaluated by agar well diffusion assay. Some of the UV-treated strains exhibited significant increase in turbidity than that of wild strain which may be attributed due to increased biomass and higher production of antibiotic. There might be possibility that mutants with lesser productivity were emerged when samples were treated with UV radiations. Further exposure of UV light may lead to the generation of revertant mutants with greater productivity. Thus, it can be concluded that the appropriated dose of UV radiation can be used for the improvement of antibiotic-producing strains. Further investigation should emphasize on the influence of the strain improvement programs in the large scale fermenter production of these antibiotics.

INTRODUCTION

Antibiotics are specific drugs, able to save lives by destroying different types of bacteria responsible for life-threatening infections. The current scenario reveals that inadequate use of antibiotics has caused the emergence of resistance among pathogenic microorganisms that result in longer, costlier and difficult treatment of infectious diseases and it is causing a very dangerous public health problem [1].

Because of the mutagenic nature of bacterial DNA, the rapid multiplication of bacterial cells, and the constant transformation of bacterial cells due to plasmid exchange and uptake, pathogenic bacteria continue to develop antimicrobial resistance, thus rendering certain antibiotics useless. Methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Pneumococcus* and vancomycin-resistant *Enterococcus faecalis* (VRE), are some of the commonplace pathogens that are proving difficult to treat effectively. More alarming consequence is the emergence of multi-drug resistance, for example, in *Mycobacterium tuberculosis* (MDRTB) strains and multi-drug-resistant (MDR) Gram-negative bacteria [2]. Thus, the search for novel antimicrobial agents is of the utmost importance [3].

Two major lines of approach can be used to attempt to reduce or overcome bacterial resistance, *viz.*, activity enhancer *i.e.* incremental improvements in existing drugs make a significant contribution to combating bacterial infections, though they are having difficulty in meeting the increasing needs of the medical community [4]. Strain improvement is important in applied microbiological research, especially in the production of clinically important antibiotics [5]. Many factors affect the level of production of a particular compound in a specific host. Classically, strain improvement has been achieved by empirical methods usually involving random mutation and subsequent screening for higher-producing progeny [6]. Thus, this program may be used to enhance the productivity. Since, actinomycetes produce approximately two-thirds of all known antibiotics of microbial origin. Over 6,000 of these compounds are produced by *Streptomyces* species and many are commercially important medicinal products used therapeutically as anti-infective (antibiotics, antifungal and antiparasitic), anticancer or immunosuppressant agents [7]. In our current study, some actinomycetes strains are evaluated for enhanced production of antibiotics after applying strain improvement program by ultra-violet (UV) radiations.

MATERIALS AND METHODS

Actinomycetes used for strain improvement

Two strains of *Streptomyces viz. S. aureofaciens* MTCC 70325 and *S. fradaei* MTCC 321 and *Micromonospora echinospora* (subsp. *pallida*) MTCC 708 were selected for present study. Strains of *Streptomyces* were revived and maintained in CM

Broth medium while *Micromonospora* was maintained in N-Z Amine broth medium.

Bacterial Strains

A total of five bacterial strains namely *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 9144, *Escherichia coli* ATCC 25922, *Salmonella typhi* MTCC 531 and *Enterococcus faecalis* ATCC 35550 were selected to evaluate their susceptibilities against test samples. These strains were revived in nutrient broth. All the above strains were procured from Microbial Type Culture Collection, IMTECH, Chandigarh.

Standard ampoules of antibiotics

Standard antibiotics used for comparative analysis were gentamicin, chlortetracycline and aureomycin. These antibiotics were purchased from local chemist.

UV treatment to wild strains

Cell suspension (10^6 CFU/ml) of the each actinomycetes strain was taken in a separate Petri-plate (90mm diameter). These plates were placed under an ultraviolet lamp (15 W, 253.7 nm wave-length) at a distance of 25 cm. Plates were irradiated for different time intervals from 10 s to 15 min. The cell suspension was kept on homogenous mixing by a magnetic stirrer during irradiation. Treated and untreated cell suspensions were then subjected to shake-flask fermentation for the production of respective antibiotic.

Fermentation of wild and mutated strains

Shake-flask fermentation was carried out in a 1 lt. conical flask with 350 ml of the respective culture medium. The flasks were separately inoculated with 5 ml of treated and untreated cell suspensions and were incubated at 28^{o}C with continuous shaking at 150 rpm in shaker-incubator. Total time of incubation was kept about 4-5 days.

Harvesting and Extraction

At the end of incubation, the optical density (OD) readings of the production media were taken at two different wavelengths, *i.e.* 610nm and 595nm using standard pre-sterilized media as the blank solution. The production media were then harvested by aseptic transfer to sterile centrifuge tubes and by centrifuging at 4000 rpm for 20 min. The supernatant, thus obtained was then stored in sterile vials and was used for microbial assay while the pellet (containing cells) was kept at 4°C for future reference.

Antibiotic assay by agar well diffusion assay

Supernatants thus obtained were acidified to pH 2.0 to 2.5 with 5 N sulfuric acid and hold for about 30 min. These supernatants were then evaluated for their activity pattern against selected microorganisms by agar well diffusion assay [8]. Petri dishes (size 100 mm diameter) containing 18 ml of cool and molten Mueller Hinton Agar (MHA) (at 40°C) were seeded with 100 µl inoculum of bacterial strain (inoculum size was adjusted so as to deliver a final inoculum of approximately 1.0 x 10^8 CFU/ml). Media was allowed to solidify and then individual Petri dishes were marked for the bacteria inoculated. Wells of 6 mm diameter were cut into solidified agar media with the help of sterilized cup-borer. 100 µl of supernatant was poured in the respective well and the plates were incubated at 37°C for overnight. The experiment was performed in triplicate under strict aseptic conditions and the antibacterial activity of each extract was expressed in terms of the average of the diameter of zone of inhibition (in mm) produced by the respective supernatant at the end of incubation period.

Determination of MICs

Supernatants were further screened to determine minimum inhibitory concentrations (MICs) by standard two-fold microbroth dilution methodology given by NCCLS [9]. A stock solution of each active supernatant was serially diluted in 96-wells microtiter plate with Mueller Hinton broth to obtain a concentration ranging from 1µg/ml to 512µg/ml. A standardized inoculum for each bacterial strain was prepared so as to give inoculum size of approximately 5 x 10^5 CFU/ml in each well. Microtiter plates were then kept at 37°C for an overnight incubation. Following incubation, the MIC was calculated as the lowest concentration of the extract inhibiting the visible growth of bacterial strain using plate reader. All the chemical ingredients used in present study were of analytical grade, and were purchased from Hi Media, India.

RESULTS AND DISCUSSION

In the present research work, some selected strains of actinomycetes were collected from MTCC and were subjected to antibiotics production via shake-flask fermentation after being exposed to UV radiation at various time intervals. At the end of incubation, each actinomycetes culture was examined for turbidity analysis by UV-Vis spectrophotometry at 595nm and 610nm wave-lengths. ODs of each wild strains were compared with mutated ones (Figure 1-3). Some of the mutated strains (A1 and A6; F6 and F8), demonstrated lesser turbidity then that of wild strain and other mutated strains. These results of lesser turbidity may be attributed to some of the harmful effects of UV light. These samples further didn't show any activity against test microorganisms when evaluated by agar well diffusion assay (Table 1).

Table 1: Inhibition zones produced by different strains of actinomycetes

	Sample Type									
Test Microorganisms	**A0**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**
Bacillus subtilis	15	0	20	22	22	19	14	15	14	12
Staphylococcus aureus	16	0	19	20	19	17	0	17	15	14
Escherichia coli	15	0	22	20	18	18	0	15	0	15
Salmonella typhi	14	0	14	14	18	20	0	14	0	14
Enterococcus faecalis	12	0	13	14	16	19	0	15	0	14
	F0	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
Bacillus subtilis	16	17	17	19	17	16	0	17	0	18
Staphylococcus aureus	16	16	16	17	16	17	0	16	0	16
Escherichia coli	12	12	12	13	12	17	0	17	0	12
Salmonella typhi	13	13	12	13	13	14	0	14	0	13
Enterococcus faecalis	12	14	13	12	14	15	0	14	0	14
	M0	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**M8**	**M9**
Bacillus subtilis	15	0	0	0	0	0	16	0	0	15
Staphylococcus aureus	16	0	0	0	0	0	17	0	0	17
Escherichia coli	13	0	0	0	0	0	14	0	0	16
Salmonella typhi	13	0	0	0	0	0	12	0	0	15
Enterococcus faecalis	12	0	0	0	0	0	12	0	0	14

A= *S. aureofaciens*; F= *S. fradiae*; M= *M. pallida*
0-wild strains; 1-9-mutated cultures at different time intervals i.e. 10 sec, 20 sec, 30 sec, 45 sec, 1 min, 2 min, 5 min, 10 min, 15 min.

Table 2: Inhibition zones produced by standard antibiotics

Test Microorganisms	**Gentamycin**	**Chlortetracycline**	**Aureomycin**
Bacillus subtilis	22	24	20
Staphylococcus aureus	20	26	19
Escherichia coli	21	22	22
Salmonella typhi	18	21	18
Enterococcus faecalis	17	19	17

Some of the UV-treated strains exhibited significant increase in turbidity than that of wild strain which may be attributed due to increased biomass and higher production of antibiotic. This fact was further confirmed in agar well diffusion assay because these samples exhibited significant zone of inhibitions as compared to wild strains. These results were further confirmed while performing the two-fold micro-broth dilution in 96-well microtitre plate for determination of minimum inhibitory concentrations (MICs) of wild and UV-treated samples (Table 3).

Our results were very much in collaboration with a similar study conducted by Kralovocova *et al* [10], in which, the production of epsilon-pyrromycinone glycosides in *Streptomyces galilaeus* increased significantly with respect to the wild strain, as a result of a sequential procedure including both natural selection and treatment with mutagens (nitrous acid, UV light and gamma-irradiation).

Similarly, Fabrizio *et al*. [11] carried out a strain improvement program and selected the spontaneous mutants from the industrial strain of *Planobispora rosea*, producer of thiazolylpeptide. Significant increase in aerial mycelium and spore formation with increased production of antibiotic was observed.

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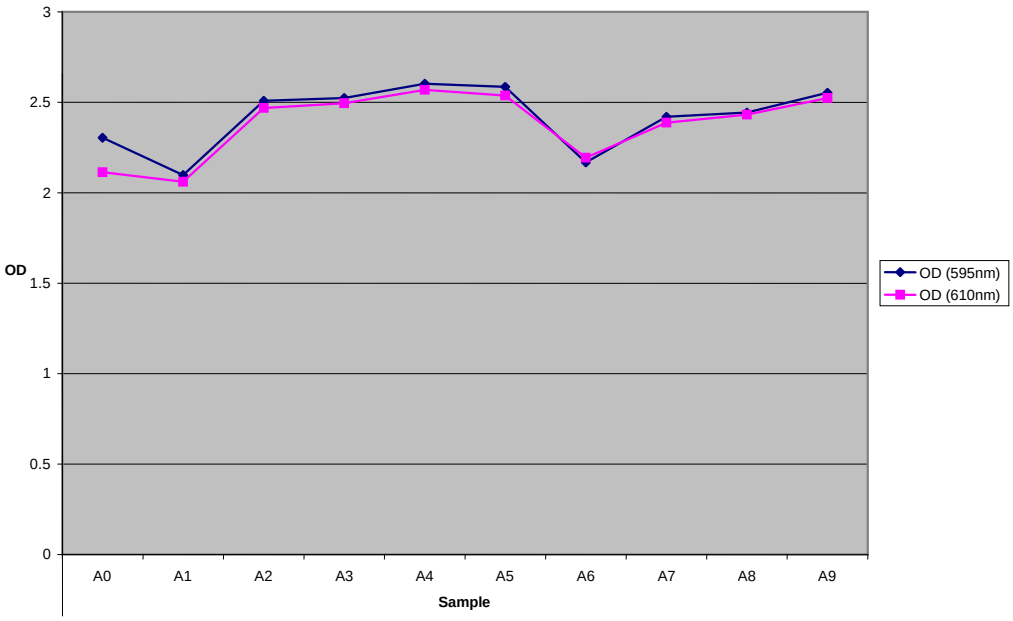

Figure 1. Optical Density for Wild & Mutated Strains of *Streptomyces aureofaciens*

Table 3: Minimum inhibitory concentrations (MICs) exhibited by different strains of actinomycetes

	Sample Type									
Test Microorganisms	**A0**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**
Bacillus subtilis	128	ND	64	64	32	64	128	128	128	128
Staphylococcus aureus	128	ND	32	64	64	64	ND	64	128	128
Escherichia coli	256	ND	64	64	128	128	ND	128	ND	128
Salmonella typhi	256	ND	128	128	128	256	ND	256	ND	256
Enterococcus faecalis	256	ND	256	512	256	256	ND	256	ND	256
	F0	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
Bacillus subtilis	128	64	64	64	64	64	ND	64	ND	64
Staphylococcus aureus	128	128	128	128	128	128	ND	128	ND	128
Escherichia coli	256	256	256	256	256	128	ND	128	ND	128
Salmonella typhi	256	256	256	128	128	128	ND	256	ND	256
Enterococcus faecalis	256	256	128	256	128	128	ND	128	ND	128
	M0	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**M8**	**M9**
Bacillus subtilis	128	ND	ND	ND	ND	ND	512	ND	ND	128
Staphylococcus aureus	128	ND	ND	ND	ND	ND	128	ND	ND	128
Escherichia coli	256	ND	ND	ND	ND	ND	256	ND	ND	128
Salmonella typhi	256	ND	ND	ND	ND	ND	512	ND	ND	256
Enterococcus faecalis	512	ND	ND	ND	ND	ND	512	ND	ND	256

All values are expressed in terms of μg/ml; ND=Not Detected

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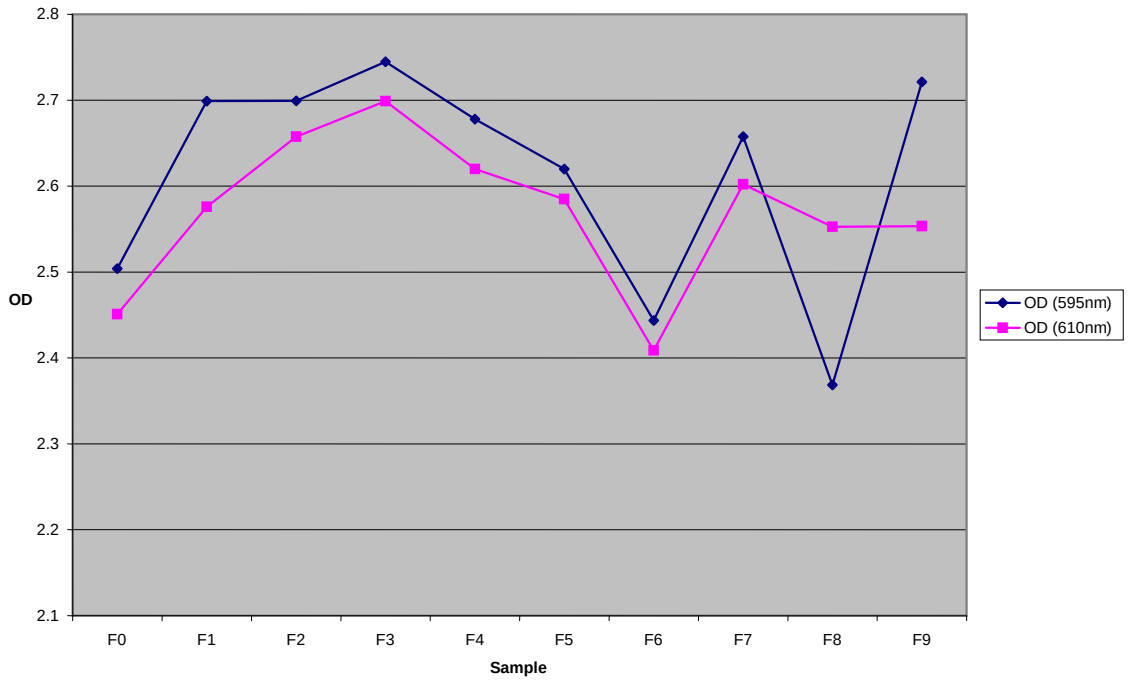

Figure 2. Optical Density for Wild & Mutated Strains of *Streptomyces fradaei*

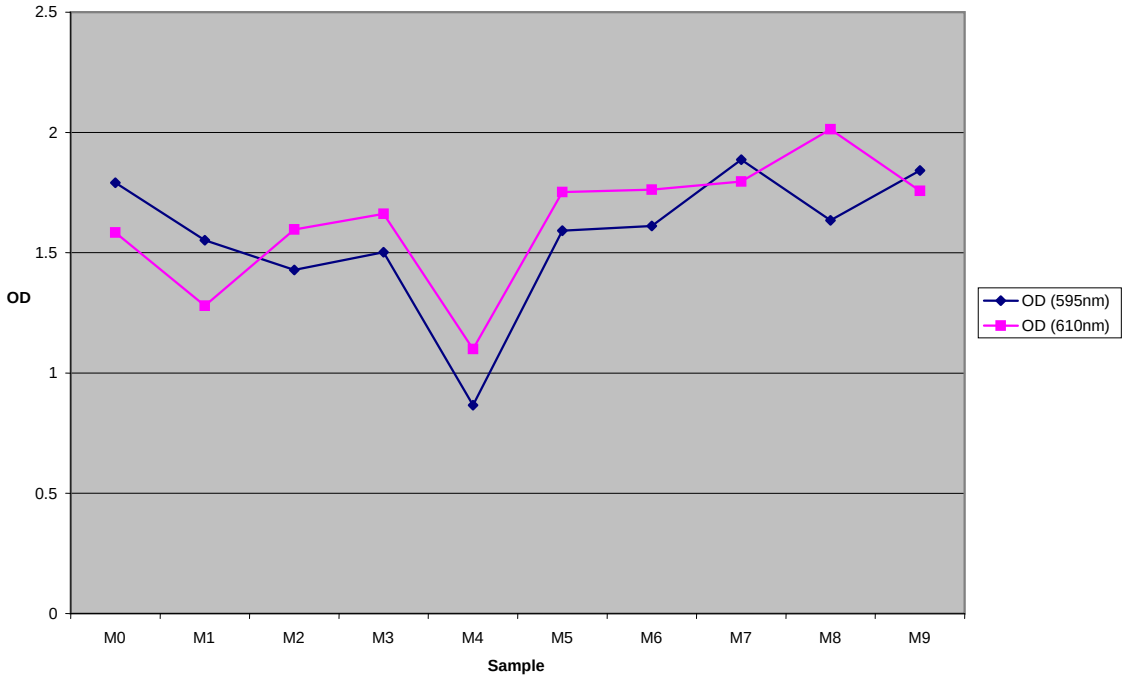

Figure 3.

Optical Density for Wild & Mutated Strains of *Micromonospora pallida*

In another study carried out by Chen *et al*. [13], a mutant, *Aspergillus* sp. CJ22-326-14, capable of showing hyper-productivity of chitosanase was isolated using successive mutation procedures UV-irradiation followed by NGT treatment of the spores.

In case of *Micromonospora*, only M6 and M9 has been showing activity against bacterial strains but most of the mutated strains were inactive. The results are the indicative of greater sensitivity of *Micromonospora* to UV lethality. Furthermore, no greater difference

between the diameter of zone of inhibitions of wild and mutated strains revealed lesser or no effect of mutation in this strain.

When compared with the standard antibiotics produced by the selected strains of actinomycetes (Table 2), UV-treated samples demonstrated almost equal activity. Antibiotics used in the present study were pure chemicals used for therapeutic purposes; purification of these UV-treated samples may give rise to improved antibiotics with maximum efficacy against microbial pathogens.

CONCLUSION

The present study clearly indicates that UV irradiation is one of the effective physical mutagen. There might be possibility that mutants with lesser productivity were emerged when samples were treated with UV radiations. Further exposure of UV light may lead to the generation of revertant mutants with greater productivity. Thus, it can be concluded that the appropriated dose of UV radiation can be used for the improvement of antibiotic-producing strains. Although optimization of fermentation media and operating parameters for the antibiotic production has been documented, some literatures are available regarding the strain improvement for antibiotic production. Further investigation should emphasize on the influence of the strain improvement programs in the large scale fermenter production of these antibiotics.

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